

## Effects of Hypophysectomy and Replacement Therapy with Bovine Growth Hormone and Triiodothyronine on the *in Vitro* Uptake of Calcium and Methionine by Scales in the Goldfish, *Carassius auratus*

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**ABSTRACT**—Using isolated scales, the involvement of bovine growth hormone (bGH) and triiodothyronine (T<sub>3</sub>) in scale formation was examined in the hypophysectomized goldfish, *Carassius auratus*. The *in vitro* uptake of calcium and <sup>35</sup>S-methionine by scales was used as indices of their calcification and matrix formation, respectively. Hypophysectomy consistently decreased the uptake of these substances with time up to 4 weeks after the operation. The direct addition of bGH or T<sub>3</sub> to the incubation medium had no recovery effect. However, the decreased uptake was restored in the incubation medium with the serum not of hypophysectomized but of sham-operated fish. The acid extract of the hepatopancreas was also effective for stimulating the uptake. The muscle, kidney, and spleen were ineffective for the extract-stimulated uptake. The hepatopancreas-stimulated uptake was also confirmed by the addition of the preincubation media in which the minced hepatopancreas was incubated with various concentrations of bGH or T<sub>3</sub> for 24 hr. These results suggest that bGH and T<sub>3</sub> stimulate scale calcification and matrix formation not via direct but via indirect sequences. A factor(s) secreted from the hepatopancreas in response to bGH and T<sub>3</sub> was considered to exert direct stimulative effects on scale formation.

### INTRODUCTION

Teleost scales have been used to determine the age of fish for a long time. They consist of two different layers, the upper osseous layer and the lower fibrillary plate, and grow at their margins by addition of the collagenous osteoid and the subsequent deposition of calcium phosphate on the osteoid. These processes are essentially conducted by scale-forming cells [1] under endocrinological control. Hypophysectomy reduced scale growth in killifish [2, 3] and goldfish [4], and replacement therapy with bovine growth hormone recovered the reduction in killifish [2]. Skeletal tissue formation is also influenced by thyroxine, which is secreted into the blood following the stimulation of thyrotropin from the pituitary. For example, Tanabe [5] reported triiodothyronine-stimulated bone formation and resorption in rainbow trout. Scales were also deteriorated by thyroxine treatment in goldfish [6]. Since these studies were conducted using *in vivo* techniques, the results include direct and indirect effects of the hormones on tissue growth. To our knowledge, the only *in vitro* study in this field was by Takagi *et al.* [7] who examined the effects of temperature, starvation, prolactin, stannocalcin, and calcitonin on calcium uptake by scales in intact goldfish and reported that only the first treatment reduced the uptake.

In this study, we examined the effect of hypophysectomy and replacement therapy with bovine growth hormone or

triiodothyronine on matrix formation and calcification in goldfish scales using an *in vitro* incubation technique. Various tissue extracts were also added to the incubation media to define the direct or indirect effect of the hormones on scale formation.

### MATERIALS AND METHODS

Goldfish, *Carassius auratus*, weighing 18–20 g were obtained from a commercial dealer and kept at 23°C under LD 12:12 for not less than 2 weeks before use. They were fed fish food pellets *ad libitum* once a day. This experiment was performed from July through November, 1992. The sex of the fish was not considered because their gonads were found to be immature at autopsy.

#### Hypophysectomy

Hypophysectomy was essentially followed by the method of Yamazaki [8]. Briefly, fish were anesthetized with MS 222 (4–6°C) and fixed sideways. A hole was drilled through the prootic bone between the first and second gill arches and the exposed pituitary was sucked out through a fine pipette. The entire procedure was conducted at 4–5°C to minimize bleeding. Sham-operated fish were subjected to only drilling. They were allowed to recover in 0.25% NaCl tap water.

#### Blood collection and calcium determination

Blood was collected from the caudal vessels by cutting the tail of the fish and draining it into glass capillaries with or without heparin treatment and then centrifuged to obtain plasma or serum. Plasma calcium concentrations were determined by atomic absorption spectrophotometry. Serum was used for addition to the incubation medium as will be described later.

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### Scale incubation

Hypophysectomized fish were kept in the tap water containing 0.25% NaCl and sacrificed after 1, 2, and 4 weeks. After a quick rinse of the body in 0.1% bleach, scales were removed from the mid dorso-ventral trunk and rinsed three times in cold Ringer solution (NaCl 135, KCl 2.5,  $\text{CaCl}_2$  3.2,  $\text{KH}_2\text{PO}_4$  2.0,  $\text{MgSO}_4$  1.0,  $\text{NaHCO}_3$  10.0, and Hepes 10.0 mM, pH 7.4) containing streptomycin (100  $\mu\text{g}/\text{ml}$ ) and penicillin (100 IU/ml). They were individually placed into wells of a tissue culture plate filled with the oxygenated Ringer solution (0.5 ml each) containing the antibiotics, 0.1% glucose, and  $^{45}\text{Ca}$  or  $^{35}\text{S}$ -methionine (NEN) in a concentration of 7.4 KBq/ml, and incubated at 23°C for 48 hr unless otherwise stated. The incubation medium was changed every 24 hr. These incubation conditions were determined by preliminary experiments. Methionine was chosen as an index of calcifiable matrix formation, because this amino acid occurred only in the  $\text{Ca}^{2+}$ -binding fraction of the otolith matrix [9]. Special attention was paid not to include regenerating or lateral-line scales.

### Radioactive counting

After incubation, scales were rinsed with agitation in distilled water for 24 hr, dried at 85°C for 12 hr, and weighed. In this case, two scales from the same individual were pooled for weighing and radioactive counting. They were placed in counting vials, solubilized in a mixture solution of  $\text{HClO}_4$  and  $\text{H}_2\text{O}_2$  (150  $\mu\text{l}$  each) at 80°C for 2 hr, and added to Scintisol EX-H (Wako Pure Chem. Co.) to determine the radioactivity using a liquid scintillation spectrophotometer (Beckman, LS6000 IC).

### Hormones and tissue extracts

Bovine growth hormone (bGH, UCB) and triiodothyronine ( $\text{T}_3$ , Sigma) were dissolved in 0.005 N NaOH (10  $\mu\text{l}$ ) and added to the incubation medium to a final concentration of 10, 100, or 1000 ng/ml. Controls received the solvent only. The muscle, kidney, spleen, and hepatopancreas were dissected out from hypophysectomized and sham-operated fish 4 weeks after operation. They were rinsed several times in the Ringer solution, homogenized in 5 M  $\text{CH}_3\text{COOH}$ , and centrifuged at  $1500\times g$  and 4°C for 30 min. Supernatants were freeze-dried and stored at -40°C until use. They were dissolved in the Ringer solution at a rate of 250 mg/ml immediately before use and centrifuged at  $1500\times g$  and 4°C for 20 min. Supernatants were used as tissue extracts. They were added to the culture medium in a final concentration of 5 mg tissue/ml. Serum obtained from these fish was also added to the incubation medium to a final concentration of 10%.

The hepatopancreas was isolated from hypophysectomized fish 4 weeks after operation, minced in the Ringer solution, and incubated with bGH or  $\text{T}_3$  (10, 100, or 1000  $\mu\text{g}/\text{ml}$ ) for 24 hr. The control incubation only received the solvent (0.005 N NaOH). Supernatants were concentrated to approximately 20  $\mu\text{l}$  and added to the incubation medium in which scales isolated from hypophysectomized fish (hypox scales) were incubated for 48 hr.

## RESULTS

Plasma calcium concentrations were approximately 2.7 mM in the sham-operated group. This level decreased to 1.8 mM 1 week after hypophysectomy ( $P < 0.01$ ) and remained low throughout an experimental period of 4 weeks (Fig. 1A). This shows that the hypophysectomy was successfully con-

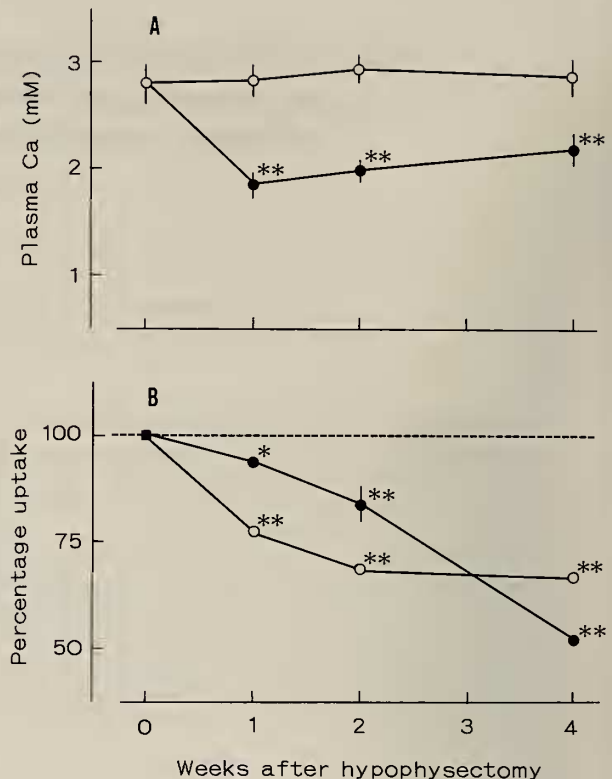


FIG. 1. Time-course related effects of hypophysectomy on plasma calcium concentrations (A, ○: sham-operated; ●: hypophysectomized) and on the *in vitro* uptake of calcium (●) and  $^{35}\text{S}$ -methionine (○) by scales (B) in goldfish. Values are means  $\pm$  SE for six fish (A) and for 18 samples (two scales/sample, B) consisting of 36 scales obtained from six individuals. At points where no error bar can be seen, the error lies within the size of the symbol. Horizontal dotted line represents the sham-operated control level (100%). \* $P < 0.05$  and \*\* $P < 0.01$  for each control.

ducted.

The *in vitro* uptake of calcium and methionine by scales was examined using the same fish. The uptake of these substances showed significant decreases ( $P < 0.05$  or  $P < 0.01$ ) 1 week after hypophysectomy, followed by further decreases to about half of the control level after 4 weeks (Fig. 1B). Time course-related uptake of calcium and methionine by scales was examined 4 weeks after hypophysectomy. The uptake of these substances increased with time up to 72 hr incubation which was the final examination time. However, the rates of uptake were significantly ( $P < 0.05$ ) less after 6 hr in the hypophysectomized group versus the sham-operated group, followed by further differences ( $P < 0.01$ ) between the two groups (Fig. 2A, B).

Incubation with various concentrations of bGH had no effect on calcium uptake by hypox scales (Fig. 3A). However, methionine uptake was stimulated ( $P < 0.05$ ) in a high bGH concentration of 1000 ng/ml (Fig. 3B). Triiodothyronine showed no effects on the uptake of these substances (Fig. 4A, B).

The addition of serum from the sham-operated fish to the incubation medium increased calcium uptake by hypox scales

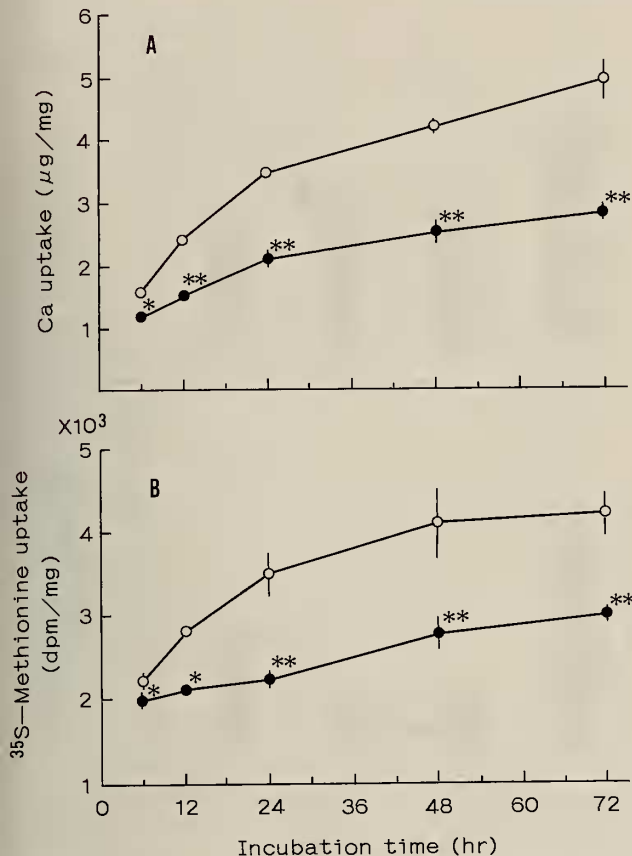


FIG. 2. Time-course related *in vitro* uptake of calcium (A) and  $^{35}\text{S}$ -methionine (B) by scales isolated from hypophysectomized goldfish. Open and closed circles represent sham-operated and hypophysectomized fish, respectively. Values are means  $\pm$  SE for 18 samples (two scales/sample) consisting of 36 scales obtained from six individuals. At points where no error bar can be seen, the error lies within the size of the symbol. \* $P < 0.05$  and \*\* $P < 0.01$  for each control.

after 24 hr ( $P < 0.05$ ) and 48 hr ( $P < 0.01$ ) compared with the addition of serum from the hypophysectomized fish (Fig. 5A). Methionine uptake was also stimulated ( $P < 0.01$ ) by the addition of the control serum after 48 hr of incubation (Fig. 5B).

Various tissue extracts were added to the incubation media to examine stimulated effects of the extracts on the uptake of calcium and methionine by hypox scales. In this experiment, hypox scales were incubated in the media containing the tissue extracts of either sham-operated (control) or hypophysectomized (experimental) fish. The extracts of the muscle, kidney, and spleen had no effect on calcium uptake by the scales (Fig. 6A). However, the addition of hepatopancreas extract significantly ( $P < 0.05$ ) stimulated the uptake. Methionine uptake was not affected by the addition of any extracts between the control and experimental incubations (Fig. 6B). However, the addition of the hepatopancreas extract resulted in the considerably high uptake of methionine in both incubations.

The minced hepatopancreas was preincubated in the

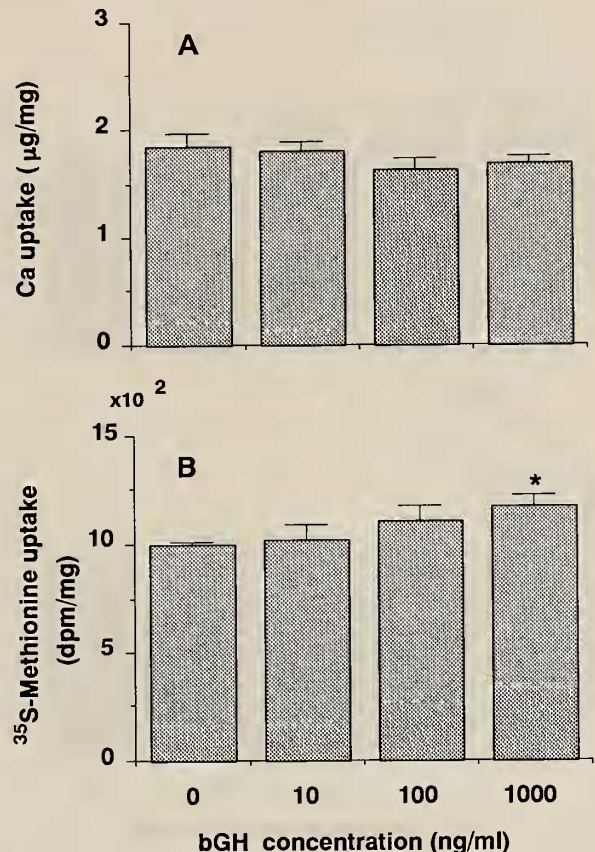


FIG. 3. Effects of various concentrations of bovine growth hormone (bGH) on the *in vitro* uptake of calcium (A) and  $^{35}\text{S}$ -methionine (B) by scales isolated from hypophysectomized goldfish. Values are means  $\pm$  SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals. \* $P < 0.05$  for the incubation without bGH.

media containing various concentrations of bGH for 24 hr and then the media were added to the incubation of hypox scales. Calcium uptake by the scales was stimulated by bGH in a concentration-dependent way up to 1000 ng/ml (Fig. 7A). Methionine uptake also increased in bGH concentrations of 100 and 1000 ng/ml (Fig. 7B).

The preincubation media in which the hepatopancreas was incubated with  $\text{T}_3$  in concentrations of 100 and 1000 ng/ml also stimulated calcium uptake by hypox scales (Fig. 8A). However, methionine uptake was stimulated only in a  $\text{T}_3$  concentration of 10 ng/ml (Fig. 8B).

## DISCUSSION

It is well documented that hypophysectomy reduced  $^{45}\text{Ca}$  uptake by scales and their growth in *in vivo* experiments [2, 4, 10]. In the present *in vitro* study, hypox scales showed consistent decreases in the uptake of calcium and methionine with time at least up to 4 weeks after hypophysectomy. The time course-related uptake of these substances was always lower in the hypox scales than the control ones. These facts may indicate that the present incubation technique is

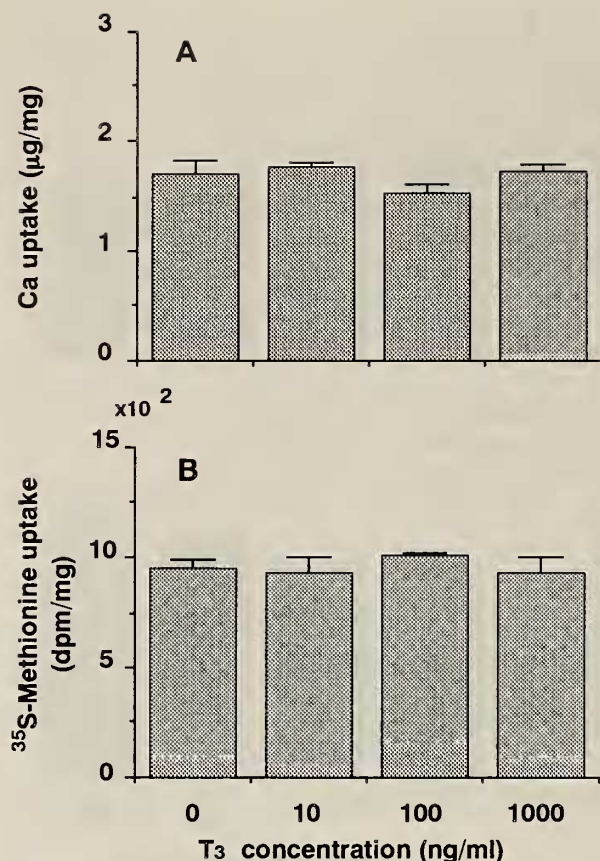


FIG. 4. Effects of various concentrations of triiodothyronine (T<sub>3</sub>) on the *in vitro* uptake of calcium (A) and <sup>35</sup>S-methionine (B) by scales isolated from hypophysectomized goldfish. Values are means ± SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals.

appropriate enough to evaluate the rates of *in vitro* uptake of calcium and methionine by scales, reflecting the physiological state of the scales. Similarly, Ottaway and Simkiss [11] used the *in vitro* uptake of glycine by scales as an indicator for the instantaneous growth rate of the scales.

Growth hormone is generally accepted to exert a secondary effect of stimulation on bone and cartilage growth via the production of an insulin-like growth factor (IGF) in the liver [12–14]. On the other hand, Isaksson *et al.* [15] and Isgaard *et al.* [16] demonstrated that GH had a direct effect on the growth of these skeletal tissues via the *in situ* production of IGF.

Ash [17] reported that the *in vitro* uptake of <sup>35</sup>S-sulfate by the gill cartilage was directly stimulated by GH in rainbow trout. However, Duan and Inui [18] found no such effect of GH on the *in vitro* uptake of <sup>35</sup>S-sulfate by the same cartilage in eels. In the present study, the direct addition of GH or T<sub>3</sub> to the incubation media did not show any positive effects on the uptake of calcium and methionine by scales except that a high concentration of GH (1000 ng/ml) stimulated the methionine uptake. Since this concentration greatly exceeds the physiological level, such a positive effect may be attributed to the pharmacological effect of the hormone. Duan

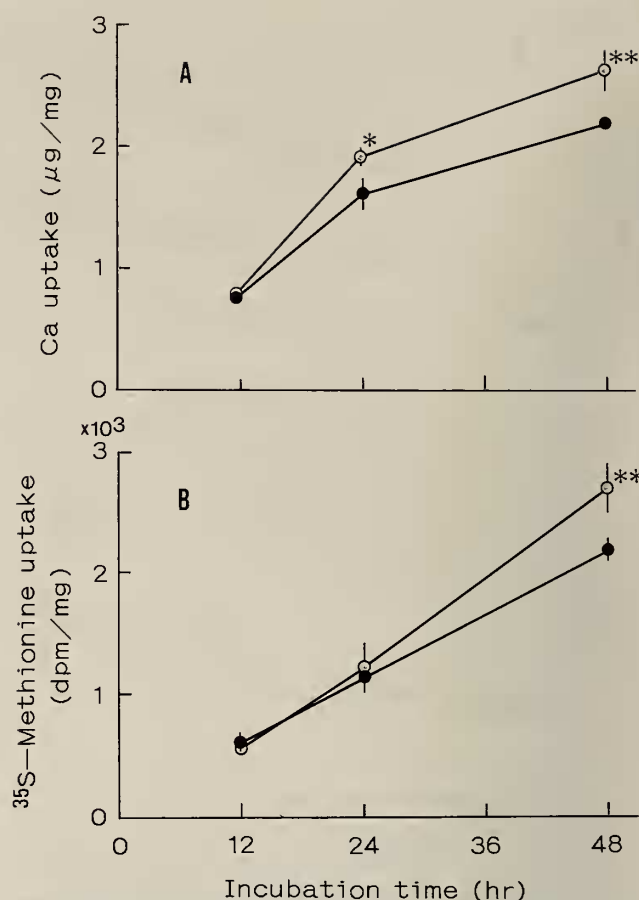


FIG. 5. Effects of serum on the *in vitro* uptake of calcium (A) and <sup>35</sup>S-methionine (B) by scales isolated from hypophysectomized goldfish. Sera were obtained from the sham-operated (○) and hypophysectomized (●) fish and added to the incubation media. Values are means ± SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals. At points where no error bar can be seen, the error lies within the size of the symbol. \**P* < 0.05 and \*\**P* < 0.01 for each control.

and Inui [18] found a time lag between the peak in the plasma level of exogenously administered GH and the stimulated uptake of sulfate by gill cartilage. Considering these results together, it seems reasonable to conclude that bGH and T<sub>3</sub> have no direct effect on scale formation.

When the serum of sham-operated fish was added to the incubation medium, it effectively stimulated the uptake of both calcium and methionine by hypox scales, while the serum of hypophysectomized fish did not. This suggests that the former serum contains a pituitary-dependent factor which stimulates scale formation. We then identified the tissue responsible for the production of the factor and found that the extract from the hepatopancreas not of hypophysectomized but of sham-operated fish increased calcium uptake by hypox scales. Methionine uptake was stimulated by both extracts. These facts suggest that the hepatopancreas is responsible for the production of the pituitary-dependent factor and that the tissue may include another factor(s) which stimulates methionine uptake by scales, independent of the

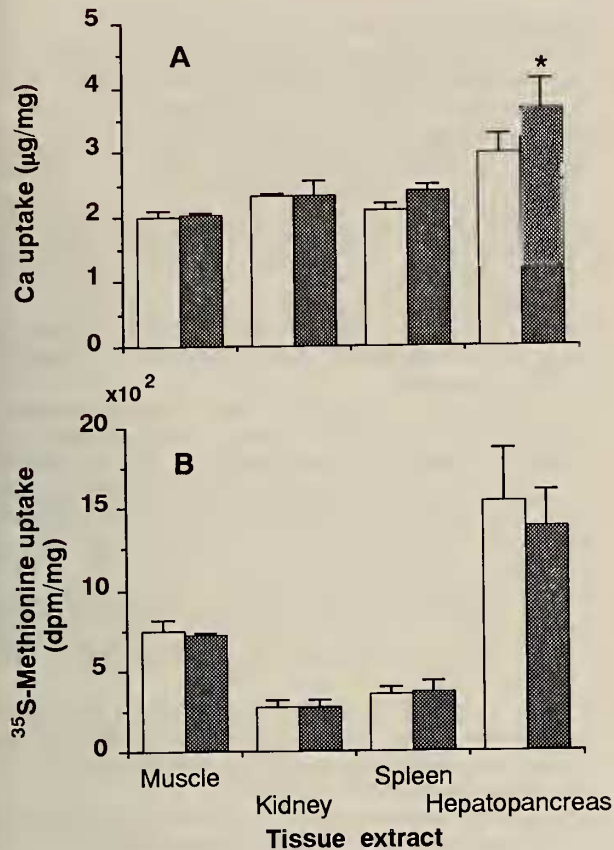


FIG. 6. Effects of various tissue extracts on the *in vitro* uptake of calcium (A) and <sup>35</sup>S-methionine (B) by scales isolated from hypophysectomized goldfish. Crude acid extracts were obtained from the four tissues in the hypophysectomized (open column) and sham-operated (shaded column) fish and added to the incubation media. Values are means  $\pm$  SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals. \* $P < 0.05$  for the hypophysectomized extract.

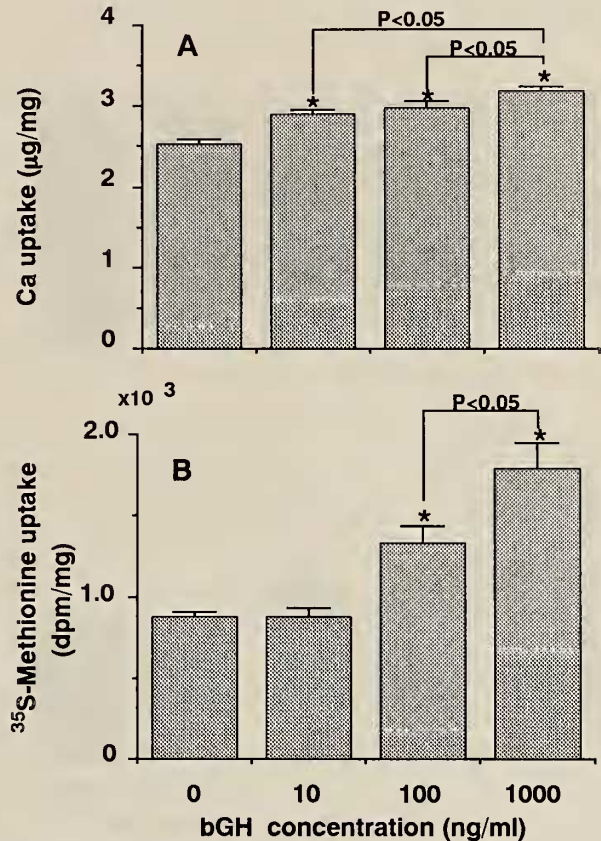


FIG. 7. Effects of the preincubation media on the *in vitro* uptake of calcium (A) and <sup>35</sup>S-methionine (B) by scales isolated from hypophysectomized fish. The hepatopancreas was incubated with various concentrations of bovine growth hormone (bGH) and the supernatants were added to the incubation media. Values are means  $\pm$  SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals. \* $P < 0.05$  for the incubation without bGH and also between the two groups indicated.

pituitary. Insulin is a candidate for the latter factor because the hepatopancreas extract may inevitably include insulin in goldfish and because insulin stimulates the *in vitro* uptake of <sup>35</sup>S-sulfate by the cartilage in eels [19].

We preincubated the minced hepatopancreas of hypophysectomized fish in the presence of bGH or T<sub>3</sub> and then the preincubated media were added to the incubation media in which the uptake of calcium and methionine by hypox scales was examined. The addition of the media induced the stimulated uptake of both substances, showing that in response to the hormones, the hepatopancreas secreted a substance(s) which had stimulative effects on scale growth. Since many studies [19–21] reported that IGF-1, which is synthesized in the liver in response to GH, stimulated <sup>35</sup>S-sulfate uptake by the cartilage, we think that the preincubation of the hepatopancreas in the presence of GH produced IGF-1, which might directly stimulate the *in vitro* uptake of calcium and methionine by hypox scales.

Triiodothyronine is known to stimulate the secretion of

GH [22, 23] and also to regulate GH receptors in the liver [24]. These facts suggest the necessity of T<sub>3</sub> for GH to produce IGF-1. However, Glasscock *et al.* [25] indicated that T<sub>3</sub> had GH-independent effects on growth stimulation in hypophysectomized rats. In the present study, T<sub>3</sub> stimulated scale formation irrespective of GH effects, suggesting the production of a new factor other than IGF in the T<sub>3</sub>-treated hepatopancreas. Further studies are needed to elucidate the sequences of the involvement of T<sub>3</sub> in scale formation.

Bovine growth hormone and T<sub>3</sub> simultaneously stimulated the uptake of calcium and methionine by scales in the present study. Skeletal tissues usually grow by the intimate interaction between inorganic and organic phases. For example, the organic matrix dually functions as a nucleator of crystals and an inhibitor of crystal growth in calcification [26]. Therefore, calcium uptake by scales will be affected by the matrix-related sequences. In the present study, however, effective concentrations of GH and T<sub>3</sub> were different between

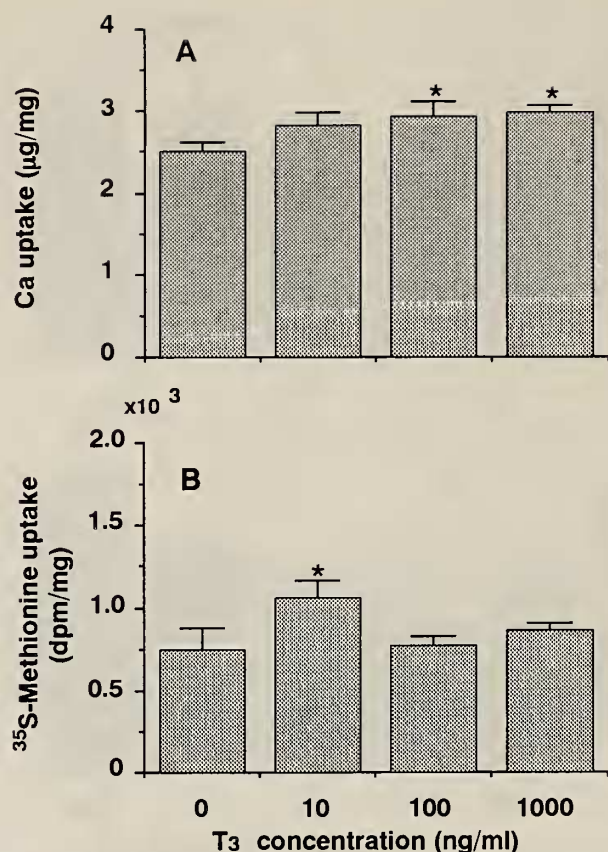


FIG. 8. Effects of the preincubation media on the *in vitro* uptake of calcium (A) and <sup>35</sup>S-methionine (B) by scales isolated from hypophysectomized goldfish. The hepatopancreas was incubated with various concentrations of triiodothyronine (T<sub>3</sub>) and the supernatants were added to the incubation media. Values are means ± SE for 15 samples (two scales/sample) consisting of 30 scales obtained from five individuals. \**P* < 0.05 for the incubation without T<sub>3</sub>.

calcium and methionine uptake. The time-related uptake of these substances was also different between calcium and methionine following hypophysectomy. These results suggest that GH and T<sub>3</sub> did not necessarily exert their stimulative effects on calcium uptake by scales via modification in the matrix formation. Matrix formation and calcification may be, at least in part, under separate control by these hormones.

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